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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

We have constructed a diphtheria toxin A (DTA) gene expression vector driven by the PSAR-PSAR promoter. The prostate-specific expression of the DTA gene specifically destroyed prostate cancere cell line LNCaP *in vitro*. Enhanced by the bystander effect, the specific expression of the DTA gene causes significant cell death in prostate cancer cell cultures, with very low background cell eradication in control cell lines. The highly specific and efficient cytopathicity of the DTA vector will potentially be valuable for systemic treatment of patients with metastatic prostate cancer. We also constructed prostate-specific lentiviral vectors and used nude mice carrying human prostate cancer cell line LNCaP as animal model (*Cancer Gene Therapy, in press).* Significant tumor regression has been demonstrated in mice injected by the prostate-specific DTA vector while no pathogenic effects have been demonstrated. Our results demonstrated that vectors that are constructed by combining our prostate tissue-specific promoter and the DTA gene offer target-specific prostate cancer cell eradication in both cell culture and *in vivo*.

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INTRODUCTION

Prostate cancer is the leading malignancy in American men, and the second leading cause of cancer deaths in men. Despite progress in early detection, surgical techniques and hormonal therapy, the ability to cure most patients with prostate cancer is limited. Metastatic and, particularly, hormone-resistant prostate cancer, has a dismal prognosis, as no current therapy offers enduring remission. Therefore, development of novel treatments is needed. Gene therapy offers a possible means by which to treat these patients. We constructed gene therapy vectors by combining the domain A fragment of the diphtheria toxin gene (DTA) with our prostate-specific promoter, the PSAR-PCPSA promoter. The constructed DTA vectors specifically eradicated prostate cancer cell line, LNCaP, in both cell culture and in nude mice. Because these vectors are tissue-specific, non-PSA-producing cells are not affected, thus, providing a potential to target and eradicate metastatic prostate cancer cells *in vivo*.

BODY

We have completed most tasks with some modifications.

Task 1. To complete the construction of the Lux gene expression vector (months 1-6)

We used the methods proposed in our research plan to improve our tissue specific vector. The addition of sequences from Epstein-Barr virus (EBV) significantly increased the levels and duration of vector gene expression (Fig.1, right panel). Compared with the parental plasmid pBM-PSAR-Lux, the EBV sequence-modified vector showed approximately 50-fold higher activity under the same transfection condition, so it would seem that our original research plan to use EBV sequence tos increase tissue specificity and transcriptional activity was correct.

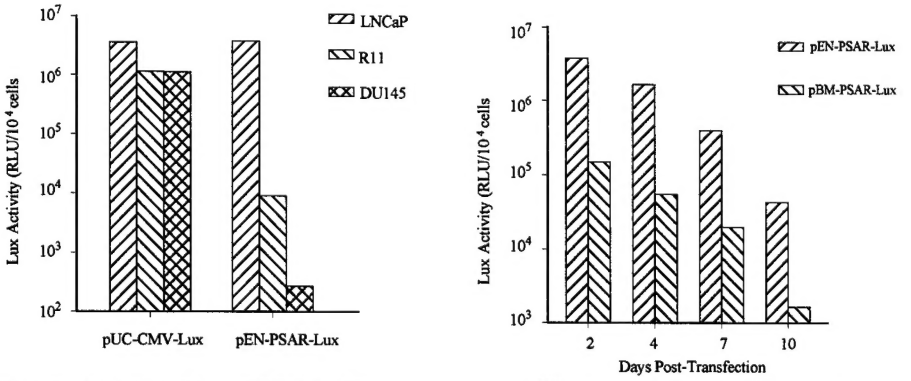

Fig. 1. Activity of the EBNA/oriP sequence modified prostate specific vector, pEN-PSAR-Lux. In the left panel, luciferase assay results from cells transfected by a vector using the modified PSAR-PCPSA promoter compared with the vector using the CMV promoter. In the right panel, gene expression duration is significantly increased with the addition of the EBNA/oriP sequences into the PSAR-PCPSA-P vector. Ten days post-transfection, the pEN-PSAR-Lux vector maintains significant expression level.

Task 2. To test the Lux expression vector in cell culture (months 7-9)

After testing more than 10 different liposomes, we found one available from Roche that is suitable for vector delivery (Fig. 1, left panel). In the transfected prostate cells, the tissue- specific promoter PSAR-PCPSA (in pEN plasmid), showed 400 to 18000-fold higher activity than in control cells. The control vector containg the CMV promoter, does not shown tissue specificity.

Task 3. To complete the construction of the Diphtheria toxin A (DTA) expression vector (month 10-11)

The DTA expression vector has been constructed and tested (see *Task 4).*

Task 4. To test the DTA vector using cell culture (months 12-18)

a. *Defining the efficiency of the DTA vector in inhibition of protein synthesis*
b. *Quantitation of the bystander effect by the DTA.*
c. *Assessing the tumor cell eradication effect by the DTA vector.*

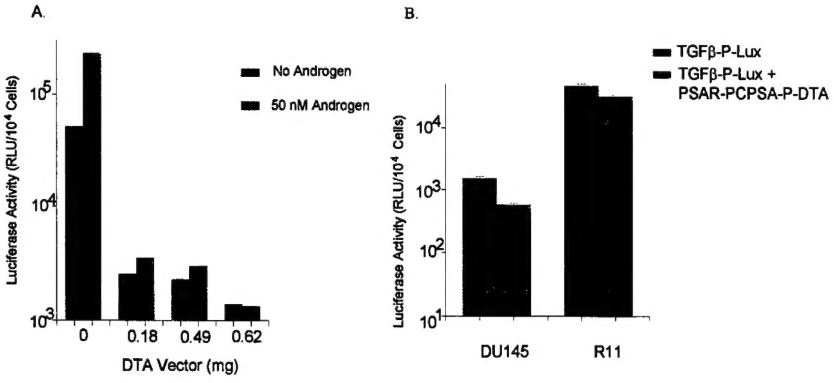

Fig. 2. The PSAR-PCPSA-P-DTA vector efficiently inhibits protein synthesis in transfected LNCaP cells. The DTA vector was co-transfected with 0.6 μg of the vector containing the Lux gene. Luciferase assays were performed two days post-transfection.
Panel A - Significant inhibition of luciferase expression was demonstrated in the LNCaP cells co-transfected with 0 to 0.62 μg of the DNA vector.
Panel B - The DTA vector demonstrated no significant effects in the R11 and DU145 cell lines when co-transfected with the TGFβ-P-lux plasmid and 0.62 μg DTA vector.

We cotransfected our DTA vector with a plasmid vector containing the *Lux* gene. Significant inhibition of the *lux* gene was demonstrated in LNCaP prostate cell line but not in the control cell lines (Fig. 2). We assessed the by-stander effect by comparing the transfection efficiency using a PSAP-EGFP (enhanced green fluorescent protein gene driven by the PSA promoter) vector, with the percentage of cell eradication in transfected cells. Our results suggested that by –stander effect is involved in DTA induced cell deaths. (Task 4, b., the figure not shown here, is published in *Cancer Gene Therapy*, 7:991, 2000). We also transfected a prostate cell line, LNCaP, and the

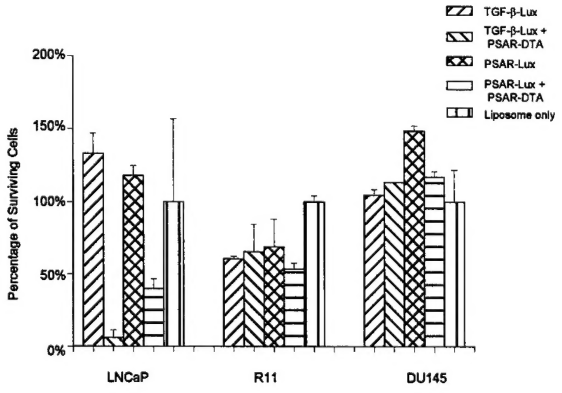

Fig. 3. Tissue-specific pathogenic effects of the prostate-specific vector carrying the diphtheria toxin A gene. Significant cell deaths in the DTA vector transfected LNCaP cells were demonstrated at day 8 post-transfection. At that day, cells from all the cultured plated transfected by various plasmid vectors were trypsinized and saved for trypan blue-staining. The cells were counted under a microscope and the percentage of surviving cells were measured. The plates with cells transfected by liposome only were assigned as 100% cell survival.

control cell lines by the prostate-specific DTA vector, and significant cell pathogenic effects were demonstrated only in the prostate cell line LNCaP (Task 4, c., see Fig. 3).

Task 5. To construct the prostate specific vector containing a modified thymidine kinase (TK) gene (month 19)

We have tested the modified TK gene in our vector, and found that it does not show any effects in eradicating prostate cancer cells *in vitro*.

Task 6. To test this gene therapy strategy using Lux expression vector to transfect human prostate tumor implanted SCID mice intravenously (IV) and intratumorally (IT) (months 20-24)

We transplanted LNCaP prostate cells with Matrigel (Fisher Scientific, Pittsburgh, PA) and control tumor cells (R11 renal cell line) into nude mice by subcutaneous injection. Significant tumors developed two to four weeks post-transplantation. However, using liposome mediated DNA transfection did not show efficient gene delivery. In such situation, we transfer our PSAR-PCPSA-P-EGFP gene expression cassette into a lentiviral vector. The prostate-specific lentiviral GFP expression vector (2 $x10^5$ pfu/0.1 ml) was injected into each tumor. Significant GFP expression was demonstrated in all injected LNCaP prostate tumors (n=10), but in none of the control R11 tumors (Fig. 4), suggesting that the prostate-specific lentiviral vector is highly tissue-specific *in vivo*.

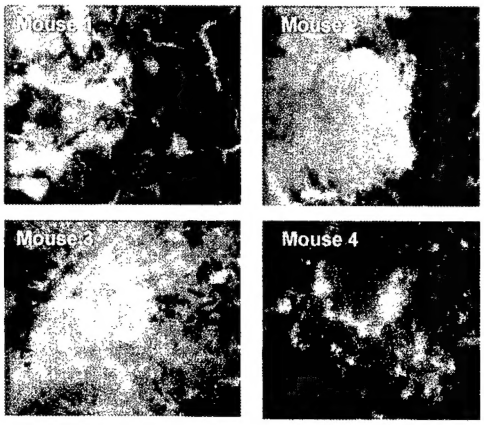

Fig. 4. Tissue-specific gene expression in LNCaP cells in nude mice. Nude mice bearing either LNCaP prostate cells or R11 renal cells were injected with $2x10^5$ pfu/0.1 ml lentiviral GFP vectors intratumorally. The animals were sacrificed five days post-injection. No GFP-positive R11 cells were detected in any injected mice bearing R11 tumors.

Task 7. To evaluate the efficacy of the DTA vector in tumor cell eradication in the SCID mice with prostate tumor xenografts (months 25-30 for Phase I, months 31-48 for phase II)

We also used the nude mouse model to test the DTA vector. When the transplanted LNCaP cells developed into tumors of approximately 0.45-cm diameter within 2 to 3 weeks (approximately 4.5(L) x 4.5(W) x 1(H) mm^3), we injected either the DTA or the EGFP control vector intratumorally. Significant tumor regression was demonstrated at 4 to 7 days post-injection of the DTA vector, whereas no tumor regression was observed in the tumors injected with the EGFP lentiviral vector (Fig.5). Our results suggest that the DTA lentiviral vector was more efficient in eradicating tumors smaller than 10 mm^3 (approximately 3 x 3 x1 mm^3). Injection of the DTA vector into these smaller tumors resulted in 100% tumor regression (Fig. 5A), as well efficient eradication of larger LNCaP tumors (approximately 4.5 x 4.5 x 1 mm^3). In the first round of experiments, we found that 5 of 7 tumors with larger

sizes showed complete response to the DTA vector (Fig. 5B). For most tumors, steady regression was demonstrated for 2 to 3 weeks. However, we also noted that DTA-induced tumor regression did not last long in some of the tumors (Fig. 5B). Some tumors resumed growth one or 2 weeks post-injection, suggesting that the growth potential of the surviving cancer cells in these animals had not actually changed.

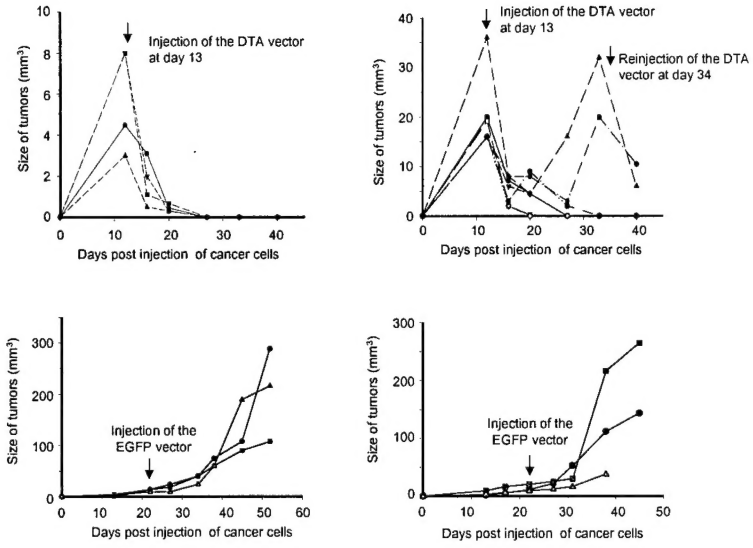

Fig. 5. Injection of the DTA or the EGFP lentiviral vector into LNCaP prostate tumor xenografts in nude mice. (A) Injection of the DTA vector into 4 tumors smaller than 10 mm^3. (B) Injection of the DTA vector into 7 tumors larger than 10 mm^3. (C) Injection of the EGFP lentiviral vector into 3 LNCaP. (D) Injection of a different viral stock of the EGFP lentiviral vector into 3 LNCaP tumors.

We repeated these experiments, using different viral stocks. In repeated testing, 10 of 15 treated tumors in nude mice completely disappeared (Fig. 5A), and 2 others had almost complete response, with very small nodules remaining. These small nodules might have been scars, because they did not grow (Fig. 6B). Only 3 of 15 tumors (one in Figure 6A, and 2 in Fig. 6B) showed significant recurrent growth. The response ratio is therefore similar to that seen in the

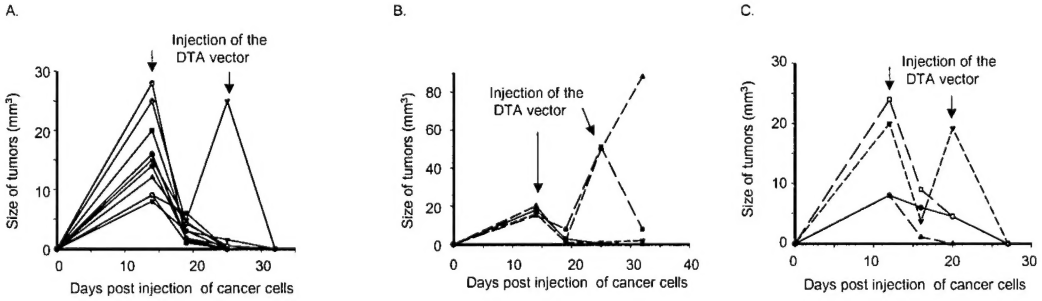

Fig. 6. A further round of experiments showing injection of the DTA lentiviral vector into LNCaP prostate cancer tumors. (A) Significant tumor regression was seen in 10 of 15 tumors. One of these 15 tumors resumed growth the second week post-injection of the DTA lentiviral vector. Re-injection of the DTA vector caused efficient regression of tumor growth. (B) Four of 15 tumors did not show a complete response to DTA treatment, but significant tumor regression was demonstrated in 3 of these 4 tumors, after a second injection of the DTA vector. However, one tumor developed resistance to the DTA vector. (C) In another experiment, after a second injection of the DTA lentiviral vector, all tumors showed complete response.

first round of experiments.

It is important to know whether the surviving LNCaP cells are resistant to the DTA vector. We injected the DTA vector into the 2 recurrent tumors shown in Fig. 5B, and the 3 tumors shown in Fig. 6A and 6B, and found that the DTA vector efficiently inhibited the growth of 4 of these 5 tumors (2 shown in Fig. 5B, one in Fig. 6A, and one in Fig. 6B), suggesting that the DTA

vector can also eradicate cancer cells in recurrent tumors. An additional test also confirmed that repetitive injection of the DTA lentiviral vector inhibited the growth of recurrent tumors (Fig. 6C). However, we noted that one of the surviving tumors showed resistance to the DTA vector (Fig. 6B), and this mechanism of resistance is currently being investigated.

Because non-specific expression of the DTA gene in cells may cause serious cytopathic effects, it is important to evaluate the safety of our DTA vector. In our previous studies using cultured cells, infection of DTA into non-PSA-producing cells did not show significant cytopathic effects, suggesting that the tissue specificity of the DTA vector confines the cytopathic effects of DTA to PSA-producing cells. To examine the safety of the DTA vector *in vivo*, we intraperitoneally injected 0.2 ml of concentrated DTA vector with a p24 count of 2-5 μg/ml into mice, and no pathogenic effects were seen in any of the 5 treated mice for 90 days, suggesting that the PSAR-PCPSA promoter can efficiently control the expression of the DTA gene *in vivo* by limiting toxic gene expression to prostate cells only, and therefore providing a safe means of treating prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS:

1. We constructed a prostate-specific DNA vector carrying the DTA gene. Significant prostate cancer eradication has been demonstrated using this vector in cell culture
2. Based on our *in vitro* data and the DTA plasmid, we constructed a lentiviral vector carrying the DTA gene controlled by the PSAR-PCPSA promoter. Our results from cell culture tests and nude mice animal model demonstrate that the DTA lentiviral vector is a very powerful vector in eradication prostate cancer in nude mice.

REPORTABLE OUTCOMES:

Two papers are published and one manuscript has been submitted (see appendix).

1. Pang S. Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. *Cancer Gene Therapy*. 2000, 7:991-996.
2. Yu D, Chen D, Chiu C, Razmazma B, Chow Y-H, and Pang S. Prostate-specific targeting using PSA promoter based lentiviral vectors. *Cancer Gene Therapy*. 2001, in press.
3. Chen D, Zheng JY, Razmazma B, Foroohar M, Yu D, Pang S. Eradication of prostate cancer by a tissue-specific DTA lentiviral vector in nude mice. Submitted.

CONCLUSIONS:

Currently there is no efficacious therapy for metastatic prostate cancer. Our long-term objective is to develop a gene therapy approach to eradicate metastatic prostate cancer cells. We have constructed a prostate tissue-specific promoter. In gene delivery vectors, this promoter restricts the therapeutic genes to express in prostate cells but not others. Therefore, therapeutic genes can be specifically expressed in prostate cancer cells, in other words, these vectors can "target" prostate cancer cells.

Prostate-specific expression of our vector has been demonstrated using either the luciferase (Lux) gene or the enhanced green fluorescent protein (EGFP) gene as reporters to quantitate tissue specificity of our vector. The gene expression level of our vector in human prostate cancer cell line LNCaP is over 400-fold higher than in non-prostate cancer cell lines or in non-PSA-producing prostate cell line DU145. Based on these data, we constructed a gene therapy vector

using the DTA gene. The DTA vector has shown very significant effect in eradicating prostate cancer cells in cell culture. Based on the results from cell culture tests, we applied our DTA vector to LNCaP prostate tumors in nude mice. Very promising results have been demonstrated. Approximately 80% treated tumors in nude mice demonstrated complete response. In these mice, tumors were completely eradicated. Other mice showed partial response to the DTA vector. Only one of 31 treated mice showed no response to the DTA vector.

We have almost completely fulfilled the tasks we proposed in our original application. Two papers have been published and one manuscript has been submitted. However, we need additional studies to improve our gene therapy approache. We need to 1) modify our vector to make it more efficient and safer, and 2) optimize gene delivery methods before apply the DTA vector to clinical trials. These two new tasks have been included in a research application submitted to DOD in March, 2001. However, by that time we submitted the research application, we have not been able to show our results of using animal model to test the DTA vector. We would appreciate your effort to attach the research progress shown in this report to the application we submitted before final review to evaluate our grant application.

REFERENCES:

APPENDICES:

Three documents (two published papers and one manuscript) are included.

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Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene

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Prostate cancer is the most commonly diagnosed malignancy in American men. Developing gene therapy for prostate cancer is important, because there is no effective treatment for patients in the advanced stages of this disease. A tissue-specific promoter to transcriptionally target cancer cells is a promising approach for gene therapy of prostate cancer. We previously constructed a prostate tissue-specific promoter based on the proximal promoter and the upstream regulatory sequence of the prostate-specific antigen gene. In the experiments described here, we modified our prostate-specific promoter to drive the A domain of the diphtheria toxin gene (DTA) in a plasmid vector. The plasmid vector can be efficiently transfected into cell lines, using a liposome-mediated transfection method. In the transfected prostate cell line, LNCaP, the DTA gene was actively transcribed, and significant inhibition of protein synthesis and cytopathic effects was observed. However, no pathogenic effects were apparent in the control cell lines. The highly specific and efficient cytopathicity of the DTA gene vector is therefore potentially useful for systemic treatment of patients with metastatic prostate cancer. **Cancer Gene Therapy (2000) 7, 991–996**

Key words: Tissue-specific promoter; prostate-specific antigen; diphtheria toxin A; prostate cancer; gene therapy.

The most difficult aspect of developing an *in vivo* approach for human cancer gene therapy is correctly targeting the specific cells of concern. Random delivery of a therapeutic gene damages normal cells in essential organs such as the liver, bone marrow, kidney, and intestine, and may subsequently cause death in treated subjects.[1]

Using a tissue-specific promoter to transcriptionally target cancer cells has shown promise.[2–4] To apply this strategy to prostate cancer gene therapy, a tissue-specific promoter is required. Although several genes have been identified that are specifically expressed in prostate cells, the most extensively studied of these is the prostate-specific antigen (PSA) gene. The PSA protein is exclusively expressed in prostate cells and has been used as a diagnostic indicator of prostate cancer. This specificity of the PSA protein suggests that the promoter of this gene may be useful for tissue-specific expression of a therapeutic gene.

The promoter and the upstream regulatory sequences of the PSA gene have been cloned by a few laboratories,[5–7] and the tissue specificity has been repeatedly confirmed. However, the cloned sequences in most cases did not show high transcriptional activity.[5–8] Increased promoter activity for a potent therapeutic gene may be necessary for a gene therapy vector to be effective.

To obtain a promoter with higher activity, we cloned the PSA promoter and upstream regulatory sequences from the cancer cells of a prostate cancer patient with extremely high concentrations of PSA in the blood (~8000-fold higher than normal). Sequence analysis revealed that there were mutations in the promoter and in the upstream regulatory sequences.[9,10] In our previous studies, we found that the combination of the 550-bp PSA promoter and the 822-bp PSA regulatory sequence together provided the highest levels of activity in driving the expression of transgenes. The activity of the patient-derived sequences was 5–50% of that of the promoter of cytomegalovirus (CMV) early genes (CMV promoter) in the prostate cell line, LNCaP. Using electroporation to transfect the promoter into the cells, the constructed promoter showed ~1000-fold tissue specificity.

We hypothesized that with such a tissue-specific promoter to drive a therapeutic gene (such as a toxic gene), we would be able to specifically kill prostate cancer cells. To increase the efficiency of gene delivery, we tested several liposomes for transfection, using vectors containing either firefly luciferase or enhanced green fluorescent protein (EGFP) as a reporter gene. With confirmed tissue specificity and gene expression efficiency of our prostate-specific promoter, we tested the potent cytotoxic diphtheria toxin A (DTA) gene for its utility as a therapeutic gene. Our results demonstrated that the

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DTA gene, driven by the prostate-specific promoter, efficiently and specifically eradicates prostate cancer cells.

MATERIALS AND METHODS

Plasmid construction

Plasmid constructs containing prostate-specific promoters and the luciferase gene have been described previously.[10] The plasmid containing the CMV promoter and the EGFP gene was purchased from Clontech (Palo Alto, Calif). To create a plasmid containing the PSAR-PCPSA promoter and the EGFP gene, we used a two-step procedure. First, the PSAR-PCPSA promoter was recovered from plasmid PSAR-PCPSAP-Luc,[10] which had been digested by the endonucleases *Xho*I and *Bam*HI. The promoter was then inserted into the multirestriction endonuclease sites of plasmid pNLDAT1RUEGFP (a gift from Dr. Irvin Chen, University of California Los Angeles School of Medicine), which had been digested with *Xho*I and *Bam*HI. Plasmid pNLDAT1RUEGFP is structurally similar to plasmid pHR′-CMVLacZ,[11] with deletion of the HIV-1 *tat* gene, the CMV promoter, and the *lacZ* gene and insertion of the EGFP gene. The plasmid containing the DTA gene, driven by the PSAR-PCPSA promoter, was constructed by replacing the *lux* gene in plasmid PSAR-PCPSAP-Luc with the DTA gene. Briefly, the DTA gene was recovered by polymerase chain reaction amplification, with plasmid pDT201 (purchased from the American Type Culture Collection (ATCC), Manassas, Va) serving as the template. The sequence coding the N-terminal 193-aa residues was amplified by the primers containing restriction sites for endonucleases *Hin*dIII at the 5′ end and Asp718 at the 3′ end. The resulting fragment was trimmed by digestion with *Hin*dIII and Asp718 before insertion into plasmid PSAR-PCPSAP-Luc, which had been digested by *Hin*dIII and Asp[718].

DNA transfection and androgen stimulation

Liposomes purchased from Life Technologies (Gaithersburg, Md), Promega (Madison, Wis), and Boehringer Mannheim (Indianapolis, Ind) were tested according to the methods suggested by the manufacturers. Cells (5×10^4) were plated to each well in 24-well plates 16 hours before lipofection. The culture medium was changed 3–16 hours posttransfection, according to the protocol provided by the vendor, and was changed every 2 days thereafter.

Androgen stimulation of transfected cells was performed by adding dihydrotestosterone (DHT) (50 nM) to the culture medium at 1 hour posttransfection.

Tumor cell lines and culture

The prostate cell lines LNCaP and DU145 were purchased from ATCC; the oral tumor cell line Tu139[12] was a gift from Dr. No-Hee Park (University of California Los Angeles School of Dentistry). The other cell lines used have been described previously.[10] Cells were maintained in RPMI 1640 medium with 10% fetal bovine sera from Life Technologies.

Luciferase assays, detection of EGFP-positive cells, and the cytopathic effect of DTA

Luciferase assays were performed using the kit from Promega. EGFP-expressing cells were detected under an ultraviolet (UV) microscope purchased from Olympus (Melville, NY). The pathogenic effect of the DTA vector-transfected cell culture was quantified, using trypsin to remove the cells from the plates, followed by trypan blue staining. The effect was assessed by comparing live cells from the cell culture plates transfected by the DTA vector with the cell culture plates transfected by control vectors.

RESULTS

Tissue-specific expression of the prostate-specific vector by liposome-mediated transfection

Although electroporation-mediated DNA transfection is efficient for delivering vectors into prostate cells,[10] it may not be suitable for *in vivo* gene delivery. Alternatively, liposome-mediated gene delivery has been shown to be feasible for clinical trials and *in vivo* gene delivery.[13–15] Because virus is not involved, this method does not induce an immune response against reinjection of the vector. To achieve high levels of transfection, we used luciferase and GFP genes as reporters to test liposomes from several sources. We selected the FuGENE 6 liposome (Boehringer Mannheim), which yielded satisfactory levels of transfection. Using an optimized liposome-mediated gene delivery method, we transfected the prostate cancer cell line LNCaP and control cell lines derived from renal carcinoma, non-PSA-producing prostate cancer, and oral cancers. In the PSA-producing prostate cell line LNCaP, the activity of luciferase was 200- to 1000-fold higher than that in the renal cell line, R11, or in the non-PSA-producing prostate cell line, DU145 (Fig 1). It was not surprising that our PSAR-PCPSA promoter does not work in the DU145 cell line, because this cell line does not express the endogenous PSA gene. The transcriptional activity and the tissue specificity obtained using liposome-mediated gene delivery were comparable with our previous results using electroporation as the means of gene delivery.[10]

Our previous studies indicated that the activity of our prostate-specific promoter is androgen-dependent. Because the culture medium contains fetal bovine sera, which has low concentrations of androgen, the prostate-specific promoter demonstrated strong activity in the LNCaP cell line. With the addition to the medium of 50 nM DHT, luciferase expression increased by ~10-fold, whereas no significant increase in luciferase expression was seen in the controls (Fig 1A). We compared the prostate-specific promoter side-by-side with the well-characterized CMV promoter, and the results indicated that the transcriptional activity of the prostate-specific promoter in the LNCaP cell line was ~10–50% of the activity of the CMV promoter (Fig 1B).

Eradication of the prostate cancer cells by the DTA expression vector

We inserted the modified thymidine kinase (TK) gene[16] into the prostate-specific vector. Although the modified TK gene should be at least 10-fold more potent than the wild-type TK gene, no significant cytopathic effects developed in the infected cells (data not shown). Alter-

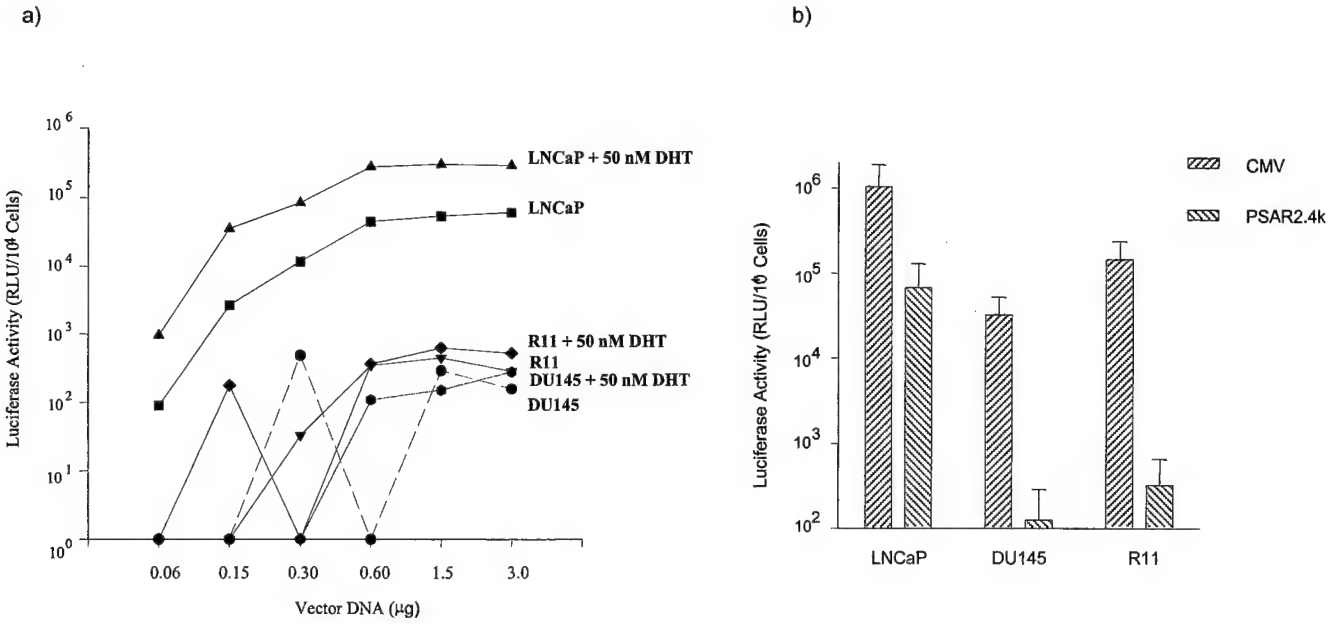

Figure 1. Transfection by a prostate-specific vector containing the luciferase gene. **A:** Plasmid PSAR2.4k-PCPSA-P-Lux[10] was used to transfect the PSA-producing prostate cell line LNCaP and the control cell lines R11 and DU145. Cells (5×10^4) were plated to each well in 24-well plates 16 hours before lipofection. Varying amounts of DNA were mixed with the FuGENE 6 liposome before being added to the cells. The androgen, DHT (50 nM), was added to one set of transfected cell lines at 1 hour posttransfection. At 2 days posttransfection, the cultured cells were lysed for luciferase assay. **B:** Comparison of the prostate-specific vector with the vector containing the CMV promoter. DNA of plasmids CMV-Lux and PSAR2.4k-PCPSA-P-Lux (1–2 μg) was mixed with appropriate amounts of the FuGENE 6 liposome (3–6 μL) before being added to each well of a 24-well culture plate. Luciferase assays were performed at 2 days posttransfection.

natively, we tested the toxic gene, DTA. The coding region of the amino terminal 193-aa residues was recovered by polymerase chain reaction, and start and termination codons were added to it. Because DTA can block protein synthesis, the effect of DTA gene expression can be evaluated by inhibition of luciferase gene expression if a luciferase gene is present. We cotransfected the DTA vector with the luciferase vector into the LNCaP cell line, and significant decreases in luciferase expression were demonstrated (Fig 2), suggesting that DTA expression in the prostate cells blocks protein synthesis.

The DTA peptide inhibits protein synthesis by binding to elongation factor-2. Although the toxicity of DTA has been widely recognized, the cytopathic kinetics of DTA have not been well-characterized. Previously, diphtheria toxin (DT) was tested and strong pathogenic effects, including induction of cell apoptosis in prostate cancer cell culture, were demonstrated.[17] It would be interesting to know whether the A subunit of DT can also perform strong pathogenic effects in the transfected prostate cells. If only transfected cells are killed by expression of DTA, the liposome-mediated transfection method would be less efficient, because only a fraction of cells can be transfected. Therefore, most cancer cells could continue to grow and become resistant to the expression of DTA.

To evaluate transfection efficiencies and to characterize expression profiles in prostate cells, we constructed a plasmid vector containing the EGFP gene driven by the prostate-specific promoter. At 4 days posttransfection,

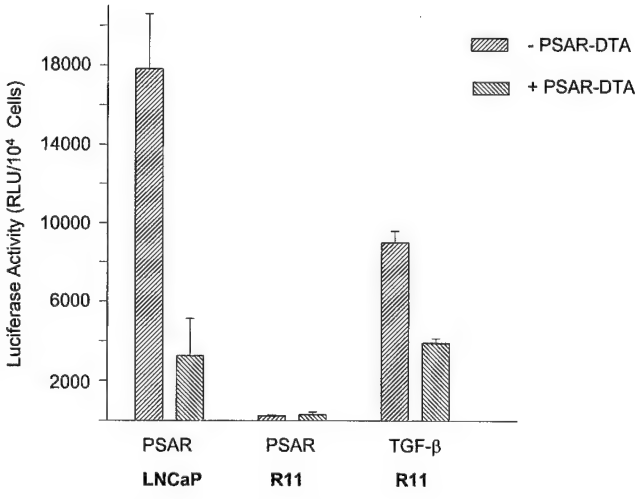

Figure 2. Inhibition of protein synthesis by expression of the DTA gene. The plasmid containing the PSAR-PCPSA promoter and the DTA gene was cotransfected either with plasmid PSAR2.4k-PCPSA-P-Lux or with the plasmid containing the transforming growth factor-β (TGF-β) promoter and the luciferase gene. Cell cultures were set up as described in Figure 1. DNA from plasmids PSAR2.4k-PCPSA-P-Lux or TGFβ-P-Lux DNA (1.2 μg) with or without 0.5–0.6 μg of PSAR-PCPSA-P-DTA plasmid was used to transfect cultured cells. Luciferase assays were performed at 2 days posttransfection.

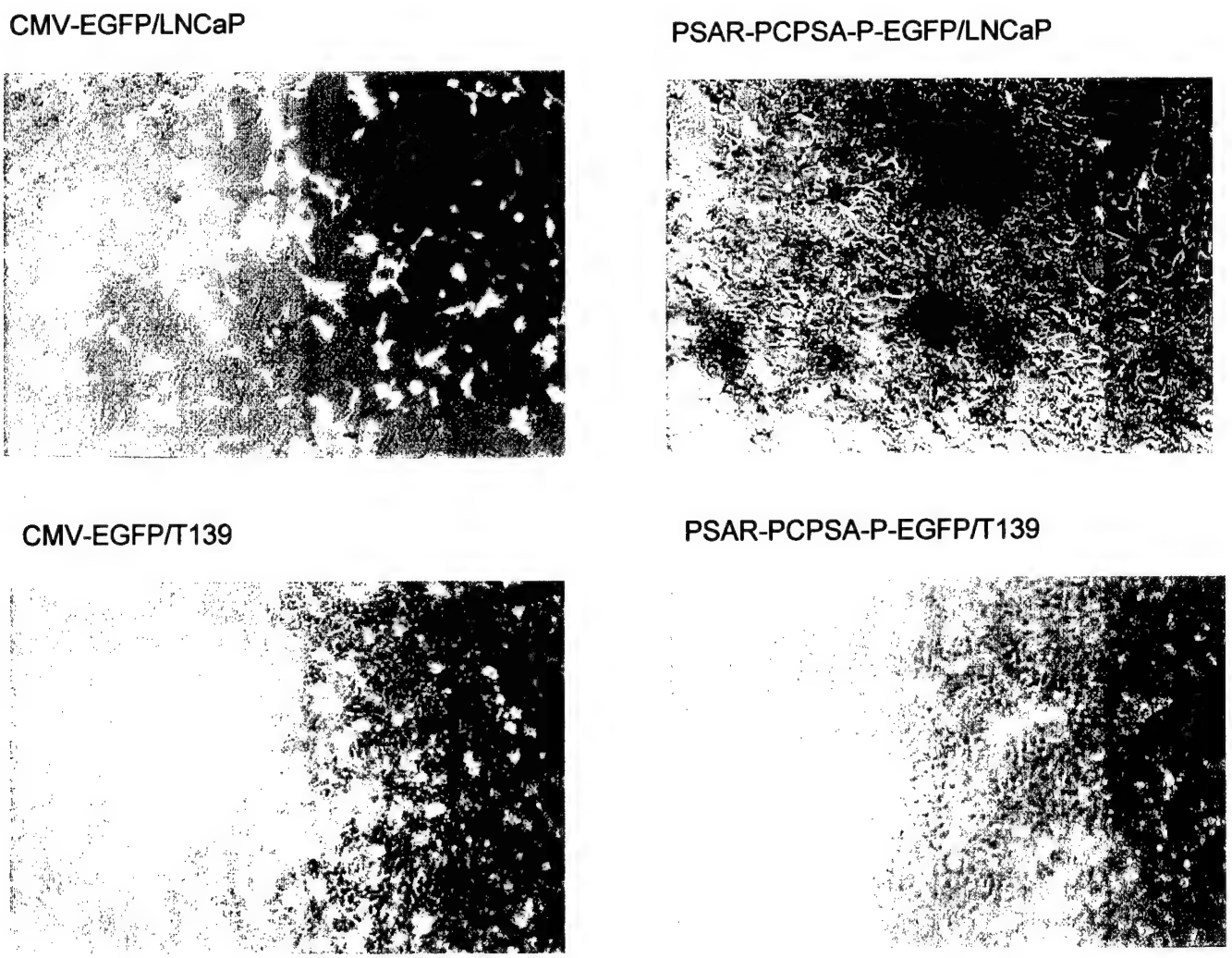

Figure 3. Plasmids containing the EGFP gene driven by either the CMV or the PSAR-PCPSA promoter were transfected into either the LNCaP cell line or the oral cancer cell line, Tu139. At 3 days posttransfection, expression of the EGFP gene could be detected under a UV microscope.

we counted positive cells under a UV microscope and found that 5–10% of LNCaP cells were transfected and had detectable levels of expression (Fig 3). The CMV vector showed higher levels of expression in the transfected LNCaP cells, with 25–50% being EGFP-positive. In the control cell lines (R11, DU145, and Tu139), the expression of EGFP was also high when these cells were transfected by the CMV vector; however, no expression was detected in these cell lines when they were transfected by the prostate-specific vector.

If only 5–10% of LNCaP cells are transfected by the prostate-specific vector, we wanted to determine whether such a rate of transfection is sufficient for cancer cell eradication by the DTA gene vector. We transfected the LNCaP and control cell lines with the DTA vector, and significant pathogenic effects were demonstrated at 7–8 days posttransfection (Fig 4). By day 8 posttransfection, >95% of cells died in the DTA-transfected LNCaP cell culture, compared with only ~10–20% in the cultures transfected by other plasmids or by liposome only. However, because only 5–10% of transfected LNCaP cells actively express the DTA protein, cell death may not be attributable simply to the expression of the DTA gene. The majority of cell deaths could have been caused by a mechanism similar to the "bystander" effect seen with the expression of the TK gene. It is also possible that DTA or other toxic factors released from the transfected cells enter the nontransfected cells, causing cell death.

To determine whether this bystander effect was occurring, we set up cell cultures in transwell plates (purchased from Fisher, Pittsburgh, Penn). The LNCaP cell line, which was placed in the lower chambers, was transfected by the DTA vector; the R11 renal cell line or the DU145 cell line was cultured in the upper chambers of the plates after transfection of the LNCaP cells. Significant cell death occurred in the transfected LNCaP cell line, but not in the upper chamber cell cultures, suggesting that the cells were not killed by factors secreted from the transfected LNCaP cells. We also added conditioned medium from the DTA vector-transfected LNCaP cell culture to the untransfected LNCaP

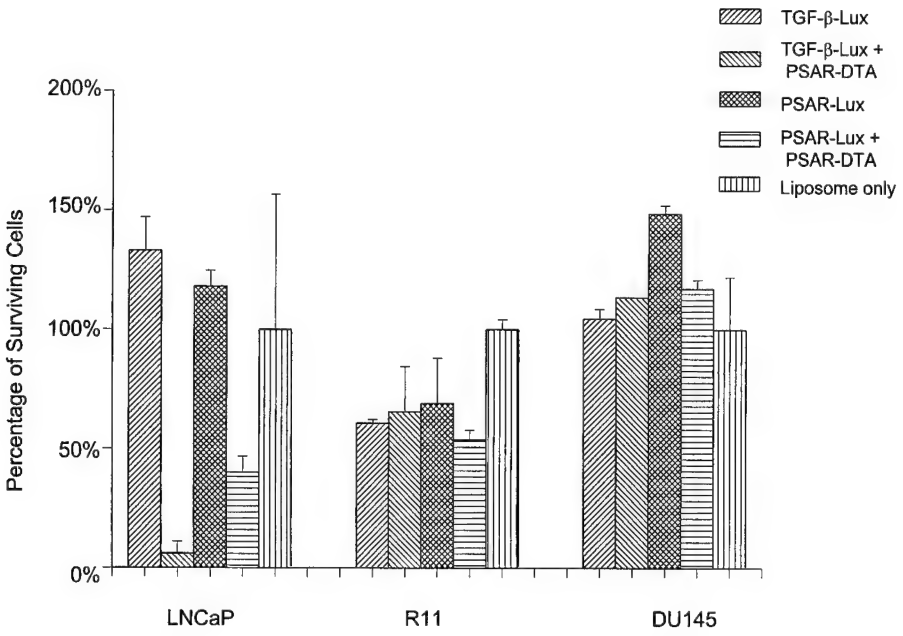

Figure 4. Tissue-specific pathogenic effects of the prostate-specific vector carrying the DTA gene. DNA transfections and cell culturing were carried out as described in Figure 2. Culture medium was changed every 3 days. Trypan blue-stained cells were counted under a microscope. The percentage of surviving cells from the plates transfected by *liposome only* was set as 100%.

cell culture, but no significant cell death occurred. These results suggest that contact of the transfected cells with the untransfected cells may be required to induce the death of untransfected cells. Previous reports suggest that the inhibition of protein synthesis by proteins such as DT induces the machinery of cell apoptosis.[17–19] The pathogenic effects of DTA may involve a chain reaction, and inhibition of protein synthesis may activate that reaction chain.

DISCUSSION

For gene therapy with a "suicide" gene, accurate targeting of cancer cells is extremely important. One strategy is to use modified retroviral vectors to specifically deliver the toxic genes into the target cells.[20,21] This approach is based on the specific binding of ligand molecules to matching cell surface receptors. The retroviral envelope protein is modified by truncation and fusion with appropriate ligand protein genes. However, this strategy is weakened by the possibility of nonspecific binding of the modified retroviral envelope protein to other cells. In addition, binding does not necessarily guarantee the entry of the vectors into the target cells. Cellular membrane proteins from the packaging cells are present in the envelope of the retroviral vector, and affect targeting accuracy. Another approach would be to use a tissue-specific promoter to obtain transcriptional targeting. To achieve tissue-specific expression, several promoters and enhancers have been tested.[2–4] To date, this approach has been impeded by "leaky expression" in inappropriate cells. The promoters that have been used for transcriptional targeting, such as the embryonic carcinoma antigen, tyrosinase, and α-fetoprotein, are not highly specific. For example, tyrosinase mRNA is also expressed in brain tissue.[22] Low levels of α-fetoprotein expression were also detected in normal liver tissue, suggesting that nonmalignant progenitor liver cells express α-fetoprotein. Therefore, the identification and development of more specific promoters is crucial.

The *in vitro* experimental results we have obtained with our PSA promoter are promising. Because the PSA protein is produced only in prostate cells, the promoter is tissue-specific. Although it is not specific to cancerous cells, treating prostate cancer patients with this promoter is unlikely to cause serious side effects, because the prostate gland is not an essential organ. This vector only targets prostate cancer cells that produce PSA mRNA, so it would not benefit those patients whose prostate cancer cells do not produce PSA mRNA. Our previous studies have indicated that cancer cells from some patients in advanced disease stages may not produce PSA mRNA. For these patients, a different prostate tissue-specific promoter would be needed.

Because DTA kills cells by apoptosis and the cells killed by apoptosis can induce an immune response specifically against cancer cells,[23,24] expression of the DTA gene in cancer cells will not only eradicate cancer cells by direct apoptosis and the bystander effect, but will also kill by indirect effects such as activation of the immune cancer cells effector cells against metastatic cancer cells.

ACKNOWLEDGMENTS

I thank Dr. D. Yu for technique supporting, Dr. N.-H. Park for providing the oral cancer cell line Tu139, Drs. I. S. Y. Chen and D.-S. An for providing the pNLDAT1RUEGFP plasmid, and W. Aft for preparation of the manuscript. This work was supported by National Institutes of Health Grant CA66022 and by U.S. Defense Department Grant DAMD 17-99-1-9033.

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Prostate-specific targeting using PSA promoter-based lentiviral vectors

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The prostate-specific antigen (PSA) promoter is known to be highly tissue specific. Although its tissue specificity has been confirmed, its efficiency of gene transcription is significantly lower compared to known nonspecific viral promoters. These lower levels of promoter activity therefore pose a problem when developing an efficacious gene vector for prostate cancer gene therapy. Thus, selecting an appropriate therapeutic gene and vector system to carry the gene driven by the PSA promoter (PSAP) is important. In the studies described here, a human immunodeficiency virus (HIV)-1–based lentiviral vector carrying either the enhanced green fluorescent protein (EGFP) reporter or the diphtheria toxin A (DTA) gene was constructed. The results demonstrate that the PSA promoter in a lentiviral vector drives genes in prostate cells with satisfactory efficacy and specificity. The tissue-specific expression of the DTA protein efficiently eradicates LNCaP prostate cells in culture. We also infected prostate cancer cells and control cells carried by nude mice with the EGFP lentiviral vector. Significant numbers of EGFP-positive LNCaP cells were detected in all the mice bearing these tumors, but no EGFP-positive control cells were detected in any other mouse tissue. The high levels of expression in prostate cells, compared with the low levels of background expression in other cells, show that the PSAP-lentiviral vector could be a potential useful tool for gene therapy of metastatic prostate cancer. **Cancer Gene Therapy (2001) 8**

Key words: PSA; PSA promoter; DTA; prostate cancer; tissue-specific.

Prostate cancer is the second leading cause of cancer deaths in American men (American Cancer Society, 1999, Internet www.cancer.org). Surgery, radiation, and hormonal therapies are currently the standard forms of treatment for prostate cancer. However, these conventional therapies are not adequate for fighting metastatic disease, especially those cases that are hormone-refractory.[1–5] Thus, new treatments are needed to combat advanced forms of prostate cancer.

Prostate cancer cells express a well-characterized antigen, prostate-specific antigen (PSA). PSA has been demonstrated to be a sensitive and specific tumor marker for cancer screening and assessment,[6–8] and is used as an indicator of disease and response to prostate cancer therapy.[9–12] PSA has highly restricted tissue distribution, and is expressed in normal epithelial cells of the prostate gland, which is the cell type in which most prostate tumors arise. Although the role of PSA in normal and neoplastic prostate cells is not well understood, the regulation machinery that enables PSA to be specifically expressed in prostate cancer cells could be very useful for constructing a tissue-specific vector to target metastatic cells.

The PSA promoter contains two sequence elements, one of approximately 550 base pairs (bp) in length flanking the PSA gene, and the other, approximately 800 bp, located 3.9 kb upstream of the 550-bp element. The PSA promoter has been extensively studied, and was cloned either from cell lines or patient samples.[13–19] The PSA promoter sequence was isolated from a prostate cancer patient with high levels of serum PSA. The transcriptional activity and tissue-specific expression of this promoter were characterized, using the cytomegalovirus (CMV) immediate early promoter as a control. The results demonstrate that the patient-derived PSA promoter is highly tissue-specific, and its transcriptional activity is approximately 5–25% that of the CMV promoter, depending on the conditions for cell growth and concentration of androgen (e.g., dihydrotestosterone) used.[18,20]

A DNA transfection method was used to deliver PSA promoter–based gene expression cassettes into prostate cells. Although this method is very useful for characterizing transcriptional potency and tissue-specific expression of this promoter, we found that only 5–20% cells were transfected.[20] Construction of a gene delivery vector with high efficacy is necessary for therapeutic applications.

The PSA promoter, along with a reporter gene, was inserted into a first-generation adenoviral vector. Gene delivery was significantly improved; however, the tissue specificity of the PSA promoter was decreased from over 400-fold (comparing transfection of the PCR-producing prostate cells with transfection of other cell lines with plasmid vectors) to approximately 20-fold or even lower,[21–23] which impedes usage of the constructed

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adenoviral vector, as a highly specific gene expression vector is essential for targeting metastatic prostate cancer cells *in vivo*. In such a context, testing other vectors is necessary. We tested human immunodeficiency virus (HIV)-1–based lentiviral vectors, which have demonstrated several advantages: (1) the promoter in lentiviral vectors is very weak in the absence of HIV-1 Tat and Rev proteins, so the tissue specificity of the inserted gene expression cassette may not be affected; (2) the essential sequences required for viral packaging are short, so more than 8 kb of DNA can be inserted; (3) these vectors infect nondividing cells; and (4) these vectors can be pseudotyped with the vesicular stomatitis virus envelope G glycoprotein (VSV-G) or other membrane proteins to increase vector infectivity and specificity. In the studies described here, we used the VSV-G pseudotyped lentiviral vector. Because VSV-G allows viral particles to bind to membrane phospholipids of target cells rather than to specific receptors, rates of infectivity are high, with a wide target spectrum.[24] The patient-derived PSA promoter was inserted into a plasmid containing the HIV-1 long terminal repeats (LTR) and the virus package signal, and either the enhanced green fluorescent protein (EGFP) (Clontech, Palo Alto, CA) or the diphtheria toxin A (DTA) gene was then inserted downstream of the PSA promoter in the plasmid. Using a three-piece transfection method,[24] lentiviral vectors with viral genomes containing either the PSAP-EGFP or PSAP-DTA expression cassettes were constructed (Fig 1) and used to infect prostate and nonprostate cell lines.

MATERIALS AND METHODS

Cell lines and maintenance

The PSA-producing cell line, LNCaP, and the non–PSA-producing prostate cell line, DU145, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The R11 cell line was originally derived from a patient with renal cell carcinoma (RCC).[18] The oral tumor cell line, TU139,[37] was a gift from Dr. N.-H. Park, UCLA School of Dentistry, and HUVEC endothelial cells

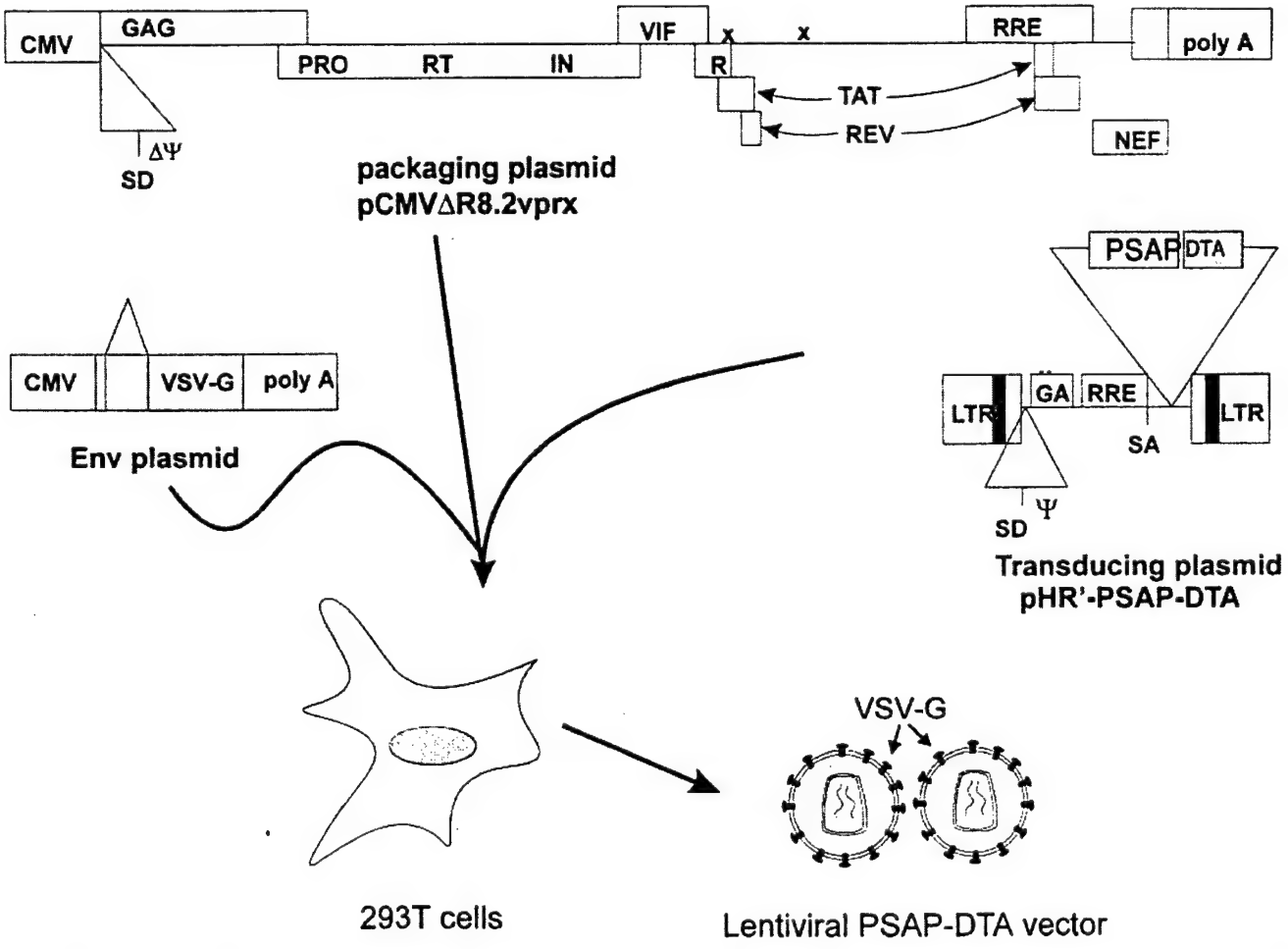

Figure 1. Construction of the lentiviral vector carrying the PSA promoter and the DTA gene. The lentiviral vector was prepared by cotransfection of three plasmids. The packaging plasmid, pCMVΔR8.2vprx, contains two point mutations, one in the *vpr* (R) coding region, and the other in the gp160 open reading frame.

were provided by Dr. L. Li, George Washington University. All cell lines except HUVEC were maintained in RPMI medium with 10% fetal bovine serum from Gibco-BRL. The HUVEC cells were maintained in EBM medium with the addition of the EGM-2 growth hormone package purchased from BioWhitaker (Walkersville, MD).

Construction and preparation of lentiviral vectors

We used a cotransfection method described previously[24] to prepare lentiviral vectors. The required plasmids, including the transduction plasmids, VSV-G (pVSV-G) and the packaging plasmid (pCMVΔR8.2vprx), were used to cotransfect 293T cells by a calcium phosphate precipitation method (Fig 1). Plasmid pCMVΔR8.2vprx carries genes required for assembly of lentiviral vectors, with deletion of the viral packaging signal and LTRs. To decrease nonspecific toxicity of the viral vectors, the *vpr* gene from the parental plasmid, pCMVΔR8.2, was mutated by oligo-directed mutagenesis. The C-terminal 64 amino acids of Vpr were deleted from the packaging plasmid, pCMVR8.2, creating plasmid pCMVΔR8.2Vprx.[38] The transfected cells were washed 16 hours posttransfection before addition of fresh medium. Lentiviral vector released from the transfected cells was collected every 24 hours from day 2 postinfection for 4 days. The collected viral stocks were passed through 0.22-μM filters to remove cell debris. The titers of virus were estimated by measuring p24 Gag protein concentrations in viral stocks. Generally, the harvested virus stocks contained approximately 100–1000 ng/mL of p24.

The control vector, a lentiviral vector carrying the CMV-EGFP expression cassette, was obtained from I.S.Y. Chen, UCLA School of Medicine. Its structure is similar to the previously reported lentiviral vector carrying the CMV-GFP expression cassette.[24,25]

Vector titration

All harvested viral vectors were subjected to p24 assays to assess viral protein content in the collected viral stocks. We also titrated the CMV-EGFP vector by infecting LNCaP cells to determine the corresponding tissue culture infection dose (TCID) with a defined p24 count. Viral aliquots with p24 counts of 3 ng were diluted up to $1/10^4$ (0.3 being the lowest amount of p24) in 200 μL of RPMI before infecting LNCaP cells (40% confluence) in 24-well plates. EGFP-positive cells were visualized and counted under a UV microscope. TCID was determined by the Karber method: $n = -\log_e(1-p)$, in which n=average number of infectious virions per cell, p=proportion of EGFP-positive cells in the culture plates, and e=2.71828. Using this equation, 50% of cells infected in a given culture (P=.5) indicated that a virus dose with an MOI of 0.693 was used $[-\log_e(1-0.5)=0.693]$. The average number of viral infectious units from these experiments was then used to convert p24 counts to the number of infecting virions in viral stocks. Our data indicate that a virus aliquot with 1 pg of p24 was equivalent to 1–10 infectious virions, which is consistent with previous reports.[39]

Infection of cells by lentiviral vectors

Cells were plated in 24-well plates at 5000 cells/well 24 hours before infection. It was expected that cells would double in number by the time of infection. Various amounts of virus equivalent to 1×10^4 to 1.2×10^6 virions (MOI 1–120) were brought up to a volume of 0.5 mL with medium before addition to each well of cultured cells. The virus-containing medium from the infected wells was removed 16 hours postinfection, and fresh medium added.

Counting EGFP-positive cells

Four days after infection by the PSAP-lentiviral EGFP vector, the infected cell cultures were checked by UV microscopy visualization to detect individual positive cells. In the wells containing more than 5% positive cells, the cells were trypsinized and washed once with phosphate-buffered saline. The total number of cells and the percentage of EGFP-positive cells were then calculated by UV microscopy.

Use of nude mice to test the PSAP-EGFP lentiviral vector in vivo

LNCaP prostate cancer cells (2×10^6) were mixed with 0.1 mL Matrigel (Fisher Scientific, Norcross, GA) before subcutaneous injection into nude mice. Tumors with a diameter of approximately 0.7 cm were formed 14 days postinjection. Mice carrying tumors derived from the control tumor cell lines R11 or PC-3 were prepared by a similar procedure, except that no Matrigel was required for growing these tumor cell lines in nude mice. Approximately 2×10^6 virions ($TCID_{50}$) in 0.1 mL of serum-free RPMI medium were injected into each mouse intratumorally. The mice were sacrificed at day 7 postinjection of the lentiviral vector. Tumors recovered from mice were minced to pieces approximately 1 mm in diameter. The EGFP-positive cells were examined under a UV microscope.

RESULTS

Tissue-specific expression of the PSAP-lentiviral vector

Cell lines LNCaP, R11, DU145, HUVEC, and TU139 were infected with a lentiviral vector containing an RNA genome carrying the CMV-EGFP expression cassette. Significant EGFP expression (>50% positive cells) was demonstrated in all tested cells infected with a titer MOI of 40 (Fig 2A), suggesting that the VSV-G pseudotyped lentiviral vector is a highly efficient vector for infecting various human cells, consistent with other previously reported results.[24,25] At very high titers of infection (MOI 40), almost 100% of the cells became EGFP-positive, with a slight pathogenic effect seen in the infected cell cultures (approximately 5–25% cell death). The cytopathic effects seen with high titers of lentiviral vector could be due to the presence of the VSV-G protein on the envelope of virions or on the small pieces of cell membrane that could pass through 0.22-μM filtration.

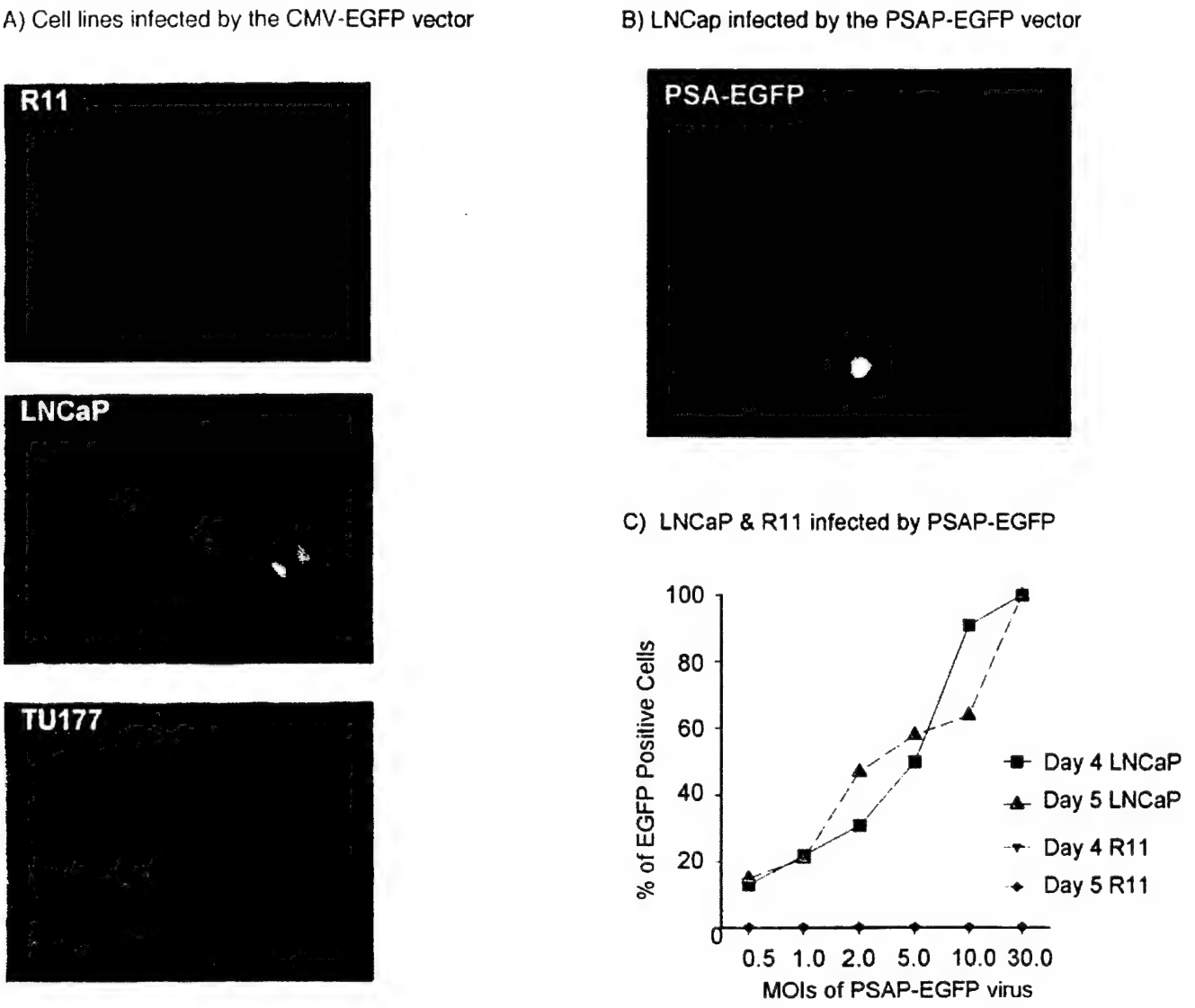

Figure 2. Tissue-specific expression of the lentiviral vector carrying the EGFP reporter gene. **A:** At day 3 postinfection, renal cell line R11, prostate cell line LNCaP, and oral cancer cell line TU177 were infected by the CMV-EGFP lentiviral vector at an MOI of 30. Other tested cell lines, including 293T, HUVEC, TU139, and HeLa-CD4, were infected by this vector at the same MOI, and showed 100% infection (not shown). **B:** At day 3 postinfection, EGFP-positive LNCaP cells infected by the PSAP-EGFP vector were present. Approximately 10% cells were strongly EGFP-positive, and more than 60% demonstrated weaker expression of EGFP. No EGFP-positive cells were present in control cell lines, except five cells in R11 (two in one well and three in another) and eight in HeLa-CD4 (3 in one well and five in another), but these percentages were less than 0.01%. Therefore, tissue specificity is approximately 10,000-fold. **C:** Dosage response of LNCaP and R11 cells infected by the PSAP-EGFP vector. Results shown are from one experiment, and were essentially identical in similar experiments.

The LNCaP prostate cell line and control cell lines were infected with the PSAP-EGFP lentiviral vector at MOIs of 0.5–30. Significant EGFP expression was demonstrated in the infected LNCaP cell line (Figs 2B and C). In other cell lines, there were almost no positive cells detected, except two or three positive cells in R11 cells infected at high MOIs (MOI 30).

Eradication of the LNCaP prostate cell line by the PSAP-DTA lentiviral vector

A lentiviral vector carrying the PSAP-DTA expression cassette was prepared and used to infect the PSA-producing cell line, LNCaP, and control cell lines R11, DU145, HUVEC, TU139, TU177, and HeLa. The LNCaP cell line was highly susceptible to infection by the PSAP-DTA vector. Significant cytopathic effects, including detachment of cells from culture plates, sphere-shaped morphological changes, and cell death, were demonstrated in the LNCaP cell line, but not in the control cell lines. When LNCaP cells were infected with the PSAP-DTA vector at an MOI of ~0.5, approximately 50% cells were killed within 24 hours. At higher MOIs, higher percentages (50–95%) of LNCaP cells were killed (Figs 3A and B). However, no significant

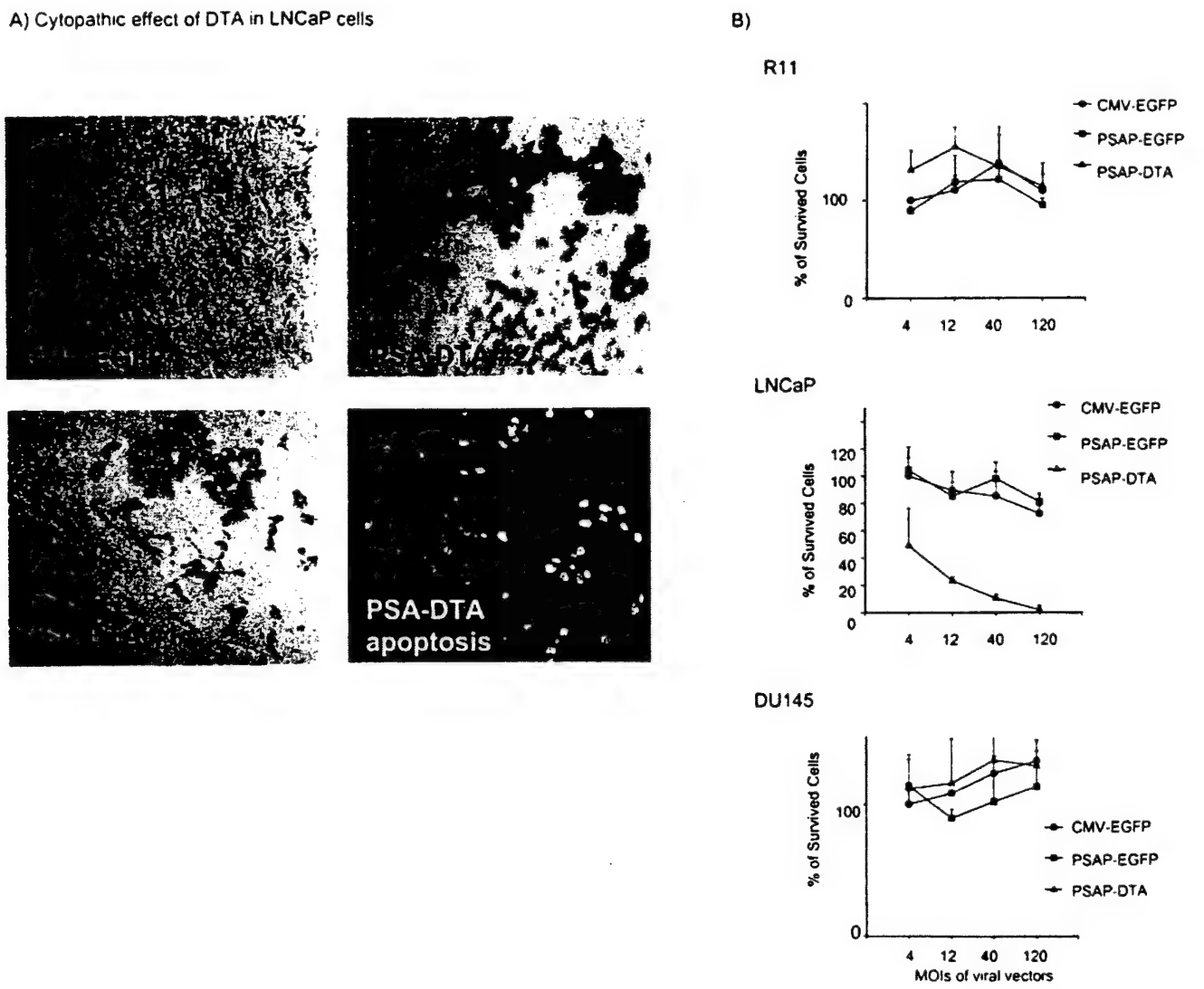

Figure 3. Tissue-specific cytopathic effects of the PSAP-DTA vector in prostate cells. **A:** No cytopathic effects were seen in the LNCaP cell line infected by the CMV-EGFP lentiviral vector, whereas significant cell death was seen when prostate cells were infected by the PSAP-DTA vector at an MOI of 40 (results from two experiments). The TUNEL assay results from the cells infected by the PSAP-DTA vector at MOI 4 suggest that some cells were killed by cell apoptosis (the cells with high light intensity in their nuclei). **B:** The dosage response of the lentiviral PSAP-DTA vector when infecting the prostate cell line and control cell lines. The proportion of cells infected by the CMV-EGFP vector at an MOI of 4 was designated as 100%. Cells were counted at 24 hours postinfection.

cytopathic effects were demonstrated in the control cell lines (Fig 3B).

Apoptosis of LNCaP cells by the PSAP lentiviral vector

Although specific inhibition of protein synthesis by DTA binding to elongation factor 2 (ELF2) has been demonstrated, the mechanism by which DTA induces cell death has not been clarified. Previous reports have suggested that DTA induces apoptosis.[20] The TUNEL staining method was used to examine infected LNCaP cells, and it was found that some cells underwent apoptosis. In the DTA vector–treated LNCaP cell culture, approximately 40% cells were detected as being apoptotic at 2 days postinfection, compared with only approximately 10% apoptotic cells in the untreated LNCaP cell culture. The high percentages of apoptotic cells partially explained the eradication effects of the LNCaP cells infected by the DTA lentiviral vector (Fig 3A).

Tissue-specific gene expression of the PSAP-EGFP lentiviral vector in nude mice

We also injected the PSAP-EGFP lentiviral vector into LNCaP tumors engrafted in nude mice intratumorally. Significant EGFP gene expression was detected in all the injected LNCaP tumors (Fig 4), whereas no EGFP-positive cells were detected in the control tumors that had been injected with the same viral stock in nude mice, including the R11 renal carcinoma cell line and PC-3, which is a non–PSA-producing prostate tumor cell line. We also tested the

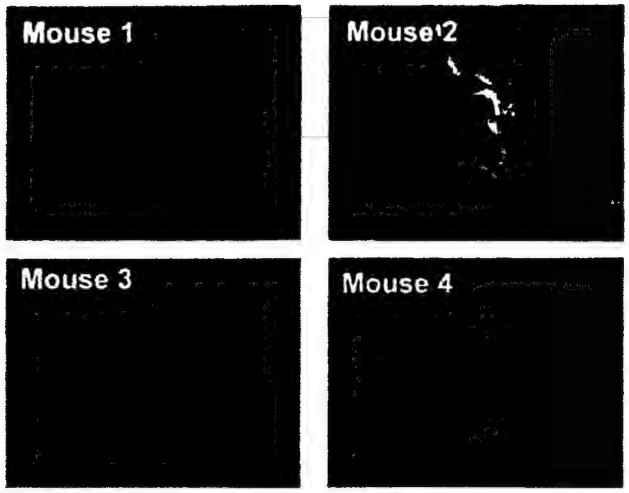

Figure 4. Infection of LNCaP tumors in nude mice. The lentiviral EGFP expression vector (2×10^8 pfu) in 0.1 mL of serum-free RPMI medium was used to infect LNCaP and control tumors in nude mice with an average size of approximately 0.7 cm in diameter. At 7 days postinfection, the animals were sacrificed, and the tumors and liver minced to small pieces of approximately 1 mm in diameter and examined under a UV microscope. The minced pieces of tumor from each animal were examined to detect EGFP-positive cells. Significant expression of EGFP was detected in all LNCaP tumors ($n=10$), whereas no EGFP-positive cells were detected in any control tumors derived from R11 and PC-3 cells ($n=15$, total) (photos not shown).

long-term pathogenic effects of lentiviral vectors by injecting nude mice with a higher dose (4×10^6 virions) of the lentiviral EGFP vector intratumorally, but found no significant pathogenic effects for three months.

DISCUSSION

Although the PSA promoter has been extensively studied, its promoter activity has not been fully characterized. Most reports have focused on responsiveness to androgen stimulation.[12–14,24–26] Absolute activity has not been clearly defined, because few studies have compared this promoter to well-characterized promoters such as the CMV promoter or the early promoter of simian virus 40 (SV40). Thus, the feasibility of using this promoter for prostate cancer gene therapy is uncertain.

The PSA promoter used in this study was isolated from a patient with prostate cancer. DNA sequence analysis indicated that the patient-derived PSA promoter contains 15 mutations when compared to the Genbank sequence, HSU37672.[15] DNA transfection demonstrated that the promoter activity is approximately 5–25% of that of the CMV promoter in a plasmid vector.[18,20] At such levels of promoter activity, the PSA promoter succeeded in driving the DTA gene in prostate cancer cells, resulting in selective cell eradication by liposome-mediated gene transfection.[20]

The drawback of using DNA vectors for cancer cell eradication is lower gene-delivery efficiency. Using liposome-mediated transfection, 5–20% cells are usually transfected in LNCaP prostate cell line cultures, but occasionally the amount can be less than 1%. Lower gene-delivery efficiency may translate to lower performance in future gene therapy clinical trials, so development of other types of vectors is desirable.

We previously inserted the 1.4-kb PSA promoter with the luciferase gene into an adenoviral vector. Although expression levels were significantly increased, tissue specificity was decreased (data not shown). Results from other research groups are similar.[21–23] Some reports suggest that inserting more positive regulatory sequences into adenoviral vectors could increase tissue specificity;[22] however, the sizes of the constructed promoters would be significantly larger (>3.5 kb), which would not allow much room for inserting therapeutic genes. In addition, the tissue specificity of the PSA promoter in this adenoviral vector, approximately 20- to 100-fold, is still much lower than that of the lentiviral vector, as shown in this report. The decreased tissue specificity would likely increase vector toxicity when a suicide gene such as DTA serves as the therapeutic gene.[29,30]

Our lentiviral vector demonstrated satisfactory levels of gene-delivery efficiency in cell culture and in a nude mouse model. It is important to note that in this vector, the PSA promoter maintained its tissue specificity. These results indicate that for efficient and tissue-specific gene expression, the lentiviral vector is superior to either adenoviral vectors or DNA plasmids. Unlike the first or second generations of adenoviral vectors, the lentiviral vector retains only a very small fraction of viral sequences. Although the retained sequences of the viral LTR could function as promoters or enhancers of transcription, the absence of supporting proteins such as Tat and Rev abrogates the promoter and enhancer functions of the LTRs. As a result, the LTRs do not interfere with the PSA promoter in the lentiviral vector, and it maintains its tissue specificity. In adenoviral vectors, many viral sequences are retained, so the opposite is true. When adenoviral promoters and enhancers are involved with the PSA promoter, its tissue specificity is significantly decreased. A new type of adenoviral vector, the helper virus–dependent (HD) adenoviral vector, has recently been reported.[31–33] The removal of adenoviral sequences that contain several nonspecific promoters and enhancers restores the specificity of tissue-specific promoters.[31] Potentially, HD virus also offers high tissue specificity when the PSAP-based gene expression cassette is inserted into this viral vector. However, thus far, no such results have been reported.

Because most prostate cancer cells grow slowly, meaning that only a small percentage of cancer cells are dividing at any given time that vectors are injected, the majority of cells, which are not dividing, will not be infected by nonlentiviral vectors. The relatively high efficiency of gene delivery by lentiviral vectors may be attributed to their capability to infect nondividing cells. Unlike other retroviruses, HIV-1 expresses genes in both dividing and nondividing cells, so the HIV-1–based lentiviral vector infects both dividing and nondividing cells.

One limitation in using the lentiviral vector for gene delivery is that it is difficult to obtain high-titer viral stocks by current cotransfection methods. In the studies described here, it was found that viral titers in medium from

cotransfected 293T cells were approximately 10^6/mL, which is about 10^4-fold lower than from adenoviral stocks.[25,34,35] Biosafety is another important consideration. Although theoretically the probability of generating a replication-competent HIV by three-piece cotransfection is rare (there is no HIV-1 viral envelope gene in the three plasmids used for cotransfection), and no report has yet demonstrated that any HIV molecules are generated from cotransfected 293T cells, the presence of HIV-1 genes and sequences during preparation of lentiviral vectors may be an important concern. Thus, a means of preparing high-titer lentiviral vectors and a safer method to generate them must be further refined in future studies.

The high infectivity of VSV-G protein–pseudotyped lentiviral vectors provides efficacious gene delivery. However, certain sensitive cells, including germline cells, may also be susceptible to the lentiviral vector. However, because most patients with prostate cancer are over 50 years of age, infection of germline cells may not pose a serious problem when using lentiviral vectors in treatments of prostate cancer.

The PSAR-PCPSA promoter has certain limitations in that it works only in cells producing PSA. Data from our laboratory and others have demonstrated that the metastatic cells of some patients may not produce PSA mRNA,[17,36] so it would be ineffectual in these cases. Identification of other prostate-specific genes and promoters would thus be necessary to target cancer cells in these patients.

Because lentiviral vectors are covered by an envelope that is derived from the membrane of the host cells, potentially we can prepare lentiviral vector from cells derived from patients. The lentiviral vectors generated would have a viral envelope with membrane proteins almost identical to the cell membrane of the patient, except for the presence of VSV-G. We expect that immunoresponse against lentiviral vectors prepared from autologous cells will be much weaker compared with viral vectors from allogeneic cells. Using nonhuman primates as animal models to test the immunoresponse against lentiviral vectors will be necessary to clarify the interaction between lentiviral gene therapy vectors and the host immune system.

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27,28

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A submitted manuscript

Eradication of prostate cancer by a tissue-specific DTA lentiviral vector in nude mice

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Summary

The prostate-specific antigen (PSA) is a tissue-specific protein. The PSA promoter-based lentiviral vector has demonstrated high specificity, and is therefore potentially useful in transcriptionally targeting prostate cancer. We have inserted the diphtheria toxin A (DTA) gene into this vector. We used human LNCaP prostate xenografts in nude mice as an animal model to test the therapeutic effects of this DTA vector, and found that with a single injection of the DTA lentiviral vector into LNCaP prostate tumors, we can completely eradicate approximately 80% of tumors (11/15, 9/11 and 3/4) in the animals. The DTA vector has also shown an ability to cause tumor regression in recurrent prostate tumors. Intraperitoneal injection of the DTA lentiviral vector into nude mice elicited no pathogenic effects, suggesting that this prostate tissue-specific vector is not only efficient, but also safe in eradicating prostate cancer cells *in vivo*.

Keywords: nude mice; PSA promoter; DTA; prostate cancer; tissue-specific; *in vivo*

Introduction

Prostate cancer is the leading malignant disease in American men. Currently, there is no effective cure for patients in advanced disease stages, and development of new therapeutic modes is needed. We propose to use gene therapy as an approach to treat these patients. We constructed a prostate tissue-specific promoter by combining the PSA promoter (the promoter of the prostate-specific antigen gene) and the upstream regulatory sequence of the PSA gene (PSAR), isolated from prostate tumor tissue from a patient in an advanced disease stage.[1,2] Using reporter genes such as luciferase (Lux) or the enhanced green fluorescent protein (EGFP), this constructed promoter, PSAR-PCPSA, demonstrated greater than 1,000-fold tissue specificity in plasmid vectors.[2] This highly tissue-specific property shows great promise for transcriptionally targeting prostate cancer cells *in vivo*. Using this promoter to drive therapeutic genes in a gene delivery vector will confine expression of these genes only to prostate cells, thereby providing a safe approach to treating prostate cancer in patients.

Because a plasmid vector may not provide efficient gene delivery, it would be difficult to use this type of vector for prostate cancer *in vivo*. To increase gene delivery efficacy, our laboratory and others have tested adenoviral vectors.[3-5] In adenoviral vectors, gene delivery efficacy is greatly increased; however, tissue specificity is decreased approximately 20-fold in the adenoviral vectors tested thus far.[3-5] This decreased tissue specificity weakens the utility of adenoviral vectors for target-specific gene therapy.

In addition to headway in developing "gutless" adenoviral vectors that may overcome the issue of decreased tissue specificity of the prostate-specific promoter in classic adenoviral vectors, we have tested a lentiviral vector. Our *in vitro* and *in vivo* experiments have demonstrated that the PSAR-PCPSA-based lentiviral vector is highly tissue-specific.[6] However, although the PSAR-PCPSA lentiviral vector offers an

approach to specifically express a gene in target cells, additional factors, including an appropriate gene to inhibit or eradicate cancer cells, are also required for efficient therapeutic effects.

Our cell culture experiments have demonstrated that the DTA gene is a suitable candidate for eradicating prostate cancer cells.[6,7] However, because gene delivery efficiency is significantly lower *in vivo*, eradication of cancer cells in the *in vivo* environment would be more difficult than eradication of cancer cells in cell culture. Here we report usage of LNCaP prostate cancer cells xenografted in nude mice as an animal model in which to test the PSAR-PCPSA-DTA lentiviral vector. Injection of the DTA lentiviral vector has demonstrated significant tumor regression, suggesting that the combination of the PSAR-PCPSA tissue-specific promoter, the DTA gene and the lentiviral vector have unprecedented potential for eradicating prostate cancer *in vivo*.

Results

We found that with the addition of Matrigel, the injected LNCaP cells developed into tumors of approximately 0.45-cm diameter within 2 to 3 (approximately 4.5(L) x 4.5(W) x 1(H) mm^3). We injected either the DTA or the EGFP control vector into these tumors. Significant tumor regression was demonstrated at 4 to 7 days post-injection of the DTA vector, whereas no tumor regression was observed in the tumors injected with the EGFP lentiviral vector (Figure 1). Our results suggest that the DTA lentiviral vector was more efficient in eradicating tumors smaller than 10 mm^3 (approximately 3 x 3 x1 mm^3). Injection of the DTA vector into these smaller tumors resulted in 100% tumor regression (Figure 1A), as well efficient eradication of larger LNCaP tumors (approximately 4.5 x 4.5 x 1 mm^3). In the first round of experiments, we found that 5 of 7 tumors with the larger sizes showed complete response to the DTA vector (Figure 1B). For most tumors, steady regression was demonstrated for 2 to 3 weeks. However, we also noted that DTA-induced tumor regression did not last long in some of the tumors (Figure 1B). Some tumors resumed growth one or 2 weeks post-injection, suggesting that the growth potential of the surviving cancer cells in these animals had not actually changed.

We repeated these experiments, using different viral stocks. In repeated testing, 10 of 15 treated tumors in nude mice completely disappeared (Figure 1A), and 2 others had almost complete response, with very small nodules remaining. These small nodules might have been scars, because they did not grow (Figure 2B). Only 3 of 15 tumors (one shown in Figure 2A, and 2 in Figure 2B) showed significant recurrent growth. The response ratio is therefore similar to that seen in the first round of experiments.

It is important to know whether the surviving LNCaP cells are resistant to the DTA vector. We injected the DTA vector into the 2 recurrent tumors shown in Figure 1B, and the 3 tumors shown in Figures 2A and 2B, and found that the DTA vector efficiently inhibited the growth of 4 of these 5 tumors (2 shown in Figure 1B, one in Figure 2A, and one in Figure 2B), suggesting that the DTA vector can also eradicate cancer cells in recurrent tumors. An additional test also confirmed that repetitive injection of the DTA lentiviral vector inhibited the growth of recurrent tumors (Figure 2C). However, we noted that one of the surviving tumors showed resistance to the DTA vector (Figure 2B), and this mechanism of resistance is currently being investigated.

Because non-specific expression of the DTA gene in cells may cause serious cytopathic effects, it is important to evaluate the safety of our DTA vector. In our previous studies using cultured cells, injection of DTA into non-PSA-producing cells did not show significant cytopathic effects, [7] suggesting that the tissue specificity of the DTA vector confines the cytopathic effects of DTA to PSA-producing cells. To examine the safety of the DTA vector *in vivo*, we intraperitoneally injected 0.2 ml of concentrated DTA vector with a p24 count of 2-5 μg/ml into mice, and no pathogenic effects were seen in any of the 5 treated mice for 90 days, suggesting that the PSAR-PCPSA promoter can efficiently control the expression of the DTA gene *in vivo* by limiting toxic gene expression to prostate cells only, and therefore providing a safe means of treating prostate cancer.

Discussion

Transcriptionally targeting cancer cells with tissue-specific promoters has shown promise.[8-12] Therapeutic genes that are driven by a prostate-specific promoter have demonstrated selective expression in prostate cells, but not other cells *in vitro* and *in vivo*.[1, 2, 7,13-17] In addition to a prostate-specific promoter, an efficient and safe delivery of the gene expression cassette and a potent therapeutic gene are also necessary for targeting and treating prostate cancer cells. We previously demonstrated that efficient and tissue-specific cancer cell eradication was achievable using the combination of the PSAR-PCPSA promoter, the DTA gene and the lentiviral vector in cell culture.[7] The results reported here indicate that significant cancer cell eradication can also be achieved *in vivo*.

The concentration of our DTA vector stocks, as measured by p24 titers, was 2-5 μg/ml p24 counts. Since one pg p24 is equivalent to one virion, each 0.2-ml injection contains 4-10 x 10^5 viral vectors. If 1 cm^3 of tumor tissue contains 10^8 cells, we would estimate that the tumors of approximately 4.5 x 4.5 x 1, grown in nude mice, contained approximately 10^6 cells. Therefore, we infected the tumors at multiplicities of infection (MOIs) of 0.4 to 1.0. At these MOIs, we would expect that more than 5% of cancer cells express the DTA gene. In combination with the so-called "bystander" effect,[6,7] all cancer cells could be eradicated. Because smaller tumors contain fewer cells, the same amount of virus should result in better rates of infection, and greater tumor regression would be achieved. In our studies, we found that the DTA vector worked better in smaller tumors, supporting this hypothesis.

We expect that a significant fraction of the lentiviral vector may not be absorbed by prostate tumor cells, but could leak to capillary vessels. The leaked portion of viral vector may infect endothelial cells in blood vessels and other organs or tissues, including the liver, kidney and lung. In these tissues or organs, the DTA gene, driven by the tissue-specific promoter, would not be expressed. Thus, a tissue-specific vector may be safer for intratumoral injection. Intraperitoneal injection of the DTA vector into mice showed no pathogenic effects, suggesting that although the DTA gene is very toxic, it is safe when this gene is controlled by the PSAR-PCPSA promoter in a lentiviral vector. We are now experimenting with higher injected doses of the DTA vector into mice, to determine the maximal dose that can be used. It is possible to use systemic injections to treat metastatic prostate cancer *in vivo* if high doses of the DTA vector do not elicit significant pathogenic effects.

One of the major disadvantages of using lentiviral vectors is the low titers of virus stocks. Currently available procedures do not render highly purified and concentrated lentiviral

vectors.[18,19] In our studies, we used ultracentrifugation to prepare the DTA lentiviral vector, and obtained vector stocks with concentrations of approximately 2-5 μg /ml p24 counts. Previous reports indicated that 1 pg of p24 Gag protein counts is equivalent to 1-190 infectious viral particles. Thus, a vector stock with a 5 μg /ml p24 count may contain less than 5 x 10^7/ml viral particles. This concentration of viral vector is significantly lower than adenoviral vectors prepared in laboratories.[20-22] Such viral titers are sufficient for treating tumors smaller than 30 mm^3; however, for larger tumors or for systemic injection, these titers may not be sufficient. While the lentiviral vector is derived from HIV-1, use of the three-plasmid co-transfection method does not generate wild-type HIV-1, but it is possible that an HIV-like virus may be generated, which uses the VSV-G protein as its envelope and contains all the genes required for virus replication. However, to date, there has been no report of replicating lentiviral vectors being generated by the three-plasmid co-transfection procedure. More extensive experimentation is necessary to completely rule out the potential risks of lentiviral vectors.

Materials and Methods

LNCaP tumors in nude mice

The LNCaP prostate cancer cell line was cultured in T162 flasks in RPMI medium supplemented with 9% fetal bovine serum. The cultured cells were harvested by trypsin-EDTA digestion for 3 min at 37°C, and washed with phosphate-buffered saline (PBS). The cells were recovered by centrifugation at 1,000 rpm for 3 min with an Eppendorf desktop, then resuspended in Matrigel (Fisher Scientific, Pittsburgh, PA) at a cell density of 3.5 x 10^7/ml at 4°C. The cells were then subcutaneously injected into each nude mouse (Charles Rever Laboratory) at 5 x 10^6 cells/mouse. Visible tumors developed at approximately 7 days post-injection. When the tumors grew to an average diameter of approximately 4-5 mm (W and L), the DTA or the control viral vectors were injected into the tumors.

Lentiviral vectors

The construction, preparation and concentration of the lentiviral DTA and EGFP vectors have been previously described.[6,23,24] The crude viral suspension harvested from transfected 293T cells was quantified by measuring the levels of the Gag protein, p24, in viral stocks. The crude viral stocks were then concentrated by ultracentrifugation.[6,14] Viral vector was concentrated to 1/100th of the original volume, to approximately 2-5 μg p24/ml. The concentrated viral stocks were saved in 1.5-ml aliquots per tube in a -80°C freezer.

Injection of lentiviral vectors

The DTA or the control vector carrying the EGFP gene (0.2 ml of either) was intratumorally injected into LNCaP tumors in mice. Because repetitive freeze/thaw cycles may decrease viral infection titers at unpredictable rates, the unused viruses were discarded.

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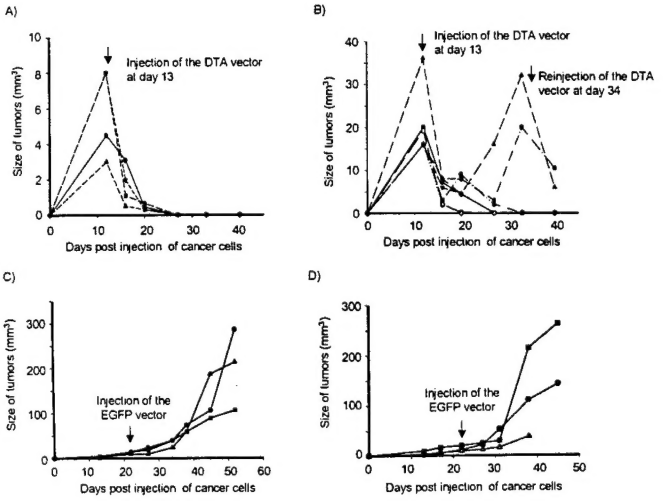

Figure 1. Injection of the DTA or the EGFP lentiviral vector into LNCaP prostate tumor xenografts in nude mice. (A) Injection of the DTA vector into 4 tumors smaller than 10 mm^3. (B) Injection of the DTA vector into 7 tumors larger than 10 mm^3. (C) Injection of the EGFP lentiviral vector into 3 LNCaP. (D) Injection of a different viral stock of the EGFP lentiviral vector into 3 LNCaP tumors.

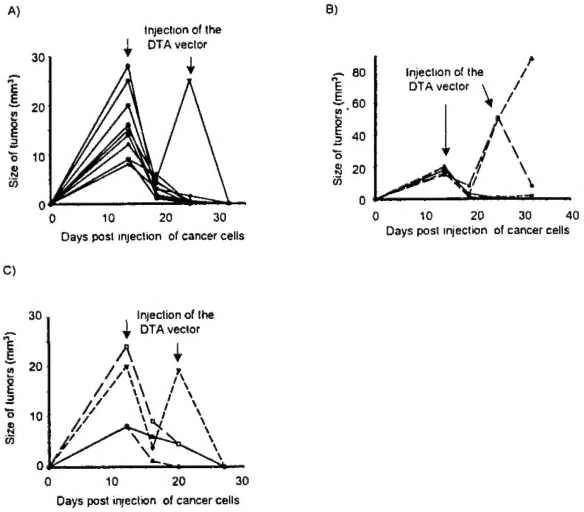

Figure 2. A further round of experiments showing injection of the DTA lentiviral vector into LNCaP prostate cancer tumors. (A) Significant tumor regression was seen in 10 of 15 tumors. One of these 15 tumors resumed growth the second week post-injection of the DTA lentiviral vector. Re-injection of the DTA vector caused efficient regression of tumor growth. (B) Four of 15 tumors did not show a complete response to DTA treatment, but